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I, John C. Decker, hereby declare:

That I possess advanced knowledge of the German and English languages. The attached translation has been translated by me and to the best of my knowledge and belief, it accurately reflects the meaning and intention of the original text.

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Components and procedure for specific inhibition of genes in eye cells

The present invention relates to a procedure to specifically or predominantly express nucleic acids, organism, host, cell, cell lines, tissue, organs, pharmaceutical compounds or medications, carriers, vectors for specific inhibition of genes in cells and tissues, especially those found within the eye on the far side of the blood-retina barrier and in the affected cells and tissues.

The human eye is an organ of extraordinary complexity, whose structures and tissue in their specific functions are so adjusted to each other that the process of seeing from impingement of the light ray on the lens up to conversion into electrical impulses and their conveyance into the areas of the brain responsible for conscious perception is optimally ensured. Particularly the tissue of the ocular fundus like the multilayer retina, in which functionally highly specialized cell types arrange conversion of light energy into electrical impulses, and also the retinal pigment epithelium (RPE) are distinguished by a very high metabolic selectivity. The active supplying of photoreceptors with nutrients from the blood stream and simultaneous removal and processing of catabolic products of the visual process is done via the RPE, which in turn is separated by Bruch's membrane from the blood vessels of the choriocapillaris. Mass transfer via the RPE and Bruch's membrane is controlled and specific and due to this functional analogy to the blood-brain barrier one speaks here of the blood-retina barrier.

The activity of numerous, often specifically expressed genes is necessary for control and implementation of the phototransduction process in the cells of the retina and of the mass transfer via the blood-retina barrier, but additionally also for maintaining the structure and functional integrity of the numerous components of tissue of the ocular fundus. This unique and highly developed system is therefore very susceptible to numerous genetic defects, that express themselves in a large phenotypic range of retinal ailments.

Besides chorioretinitis and herpes retinitis, which can be regarded as acquired forms of retinal ailments, a predominant number of retinal clinical pictures may be traced back to a genetic disposition. Among these for example are primary retinal detachment (ablatio retinae), retinoblastoma, retinal astrocytoma (Bourneville Pringle), angiomatosis retinae (Hippel-Lindau), Coats disease (retinitis exsudativa), Eales disease, retinopathia centralis serosa, ocular albinism, retinitis pigmentosa, retinitis punctata albescens, the Usher syndrome, Leber's congenital amaurosis, cone dystrophy, vitelliform macular generation (Morbus Best), juvenile retinoschisis, North Carolina macular dystrophy, Sorsby fundus dystrophy, Doyne's honeycomb retinal dystrophy (Malattia leventinese), Morbus Stargardt, Wagner's vitreoretinal degeneration and age-related macular degeneration (AMD).

While impressive progress exists in elucidating the causes of monogene retinopathies like Morbus Best or Morbus Stargardt in recent times, current knowledge about the often complex molecular metabolic interconnections which underlie the characteristic clinical picture of multifactorally caused retinal ailments, is only very meager. In the following, AMD will be gone into as a complex eye disease with genetic components and the technical problems connected therewith in relation to researching the molecular causes and development of diagnostic and pharmacological intervention strategies.

AMD, which is counted among the retinal degerations, is the most frequent cause of blindness at advanced age in the developed world. Occurrence rises from 9% of persons at an age of more than 52 years to more than 25% at over 75 years (Paetkau et al. 1978, Leibowitz et al. 1980, Banks and Hutton, 1981, Ghafour et al. 1983, Hyman 1987, Hyman et al. 1983, Grey et al. 1989, Yap and Weatherill 1989, Heiba et al. 1994).

AMD is a complex disease which is triggered by both endogenous and also by exogenous factors (Meyers and Zachary 1988; Seddon et al. 1997). Besides environmental factors, various personal risk factors like hypermetropia, light skin and iris color, high serum cholestorol levels, high blood pressure or

cigarette smoking are proposed (Hyman et al. 1983, Klein et al. 1993, Sperduto and Hiller 1986, The Eye Disease Case-Control Study Group 1992, Bressler and Bressler 1995). A genetic component as the basis of AMD was documented by various groups (Gass 1973, Piguet et al. 1993, Silvestri et al. 1994) and led to the hypothesis that the disease in appropriately genetically predisposed persons can be abetted by environmental and individouble factors.

Owing to the complexity of the clinical picture, it can be assumed that the number of previously unknown genes which, when they have mutated, contribute to susceptibility for AMD, is large.

The technical problem that underlies this invention consists now for one in that a deliberate application of agents into the eye, and especially into the ocular fundus, owing to the structure of the human eye, is expensive, linked with substantial side effects, and delayed consequences must be expected. Direct application for example by injection into the ocular fundus is very unpleasant for the affected persons, especially when treatment is required multiple times or chronically. Additionally, direct application into the bulbus is linked with substantial side effects or secondary ailments appearing at medium range like cataracts or glaucoma. Systemic application in contrast shows side effects as a rule outside the eye, the target organ – often without the agent detectable in significant quantities in the ocular fundus of the inner eye. Even with sufficient target specificity, that would keep the risk of undesired side effects with a systemic application low, this type of application remains inefficient, since the target tissue and target cells are on the far side of the blood-retina barrier, and due to the stringent activity of this barrier, the agent cannot get to its active location.

Medications currently available on the market for treatment of ophthalmological diseases are available therefore almost exclusively for treatment of clinical symptoms of the outer eye, since application via eye drops is a relatively easy possibility.

Causal therapy, especially of the rear eye sections is not possible, with the exception described above and injections involving side effects.

For another, the technical problem consists in the difficulty of applying conventional experimental strategies for identification of genes that are causal for retinal ailments, and of their validation as targets for diagnosis and for pharmacological intervention strategies. This is especially true for AMD, since with this disease the systems appear only late, as a rule in the 7th decade of life. Knowledge about pathological metabolic connections due to limited or absent function of one or more genes that perhaps cause this in AMD or in other retinal clinical pictures, does not suffice according to the prior state of knowledge for medical treatment of such ailments. Suitable animal or cell culture models for such diseases are lacking due to the complexity of the ailment and corresponding simple intervention and manipulation options in the eye.

As the essential sensory organ, the eye is protected by natural barriers (tear secretion, enzymes, transport processes, blood-retina barrier) against exterior damaging influences, but also represents a barrier for application of medications. Like the blood-brain barrier, the blood-retina barrier represents a physiological barrier for uptake of medications into the interior of the eye, and makes pharmacological therapy of ocular diseases according to the current state of the art only possible with great difficulty, if at all. This holds true for example also for the application of so-galled single-strand antisensory oligonucleotides for inhibiting the expression of goal genes, whose application in the eye makes necessary an intravitreal injection. Thus, overcoming the blood-retina barrier represents a technical problem in the therapy of ocular diseases through specific inhibition of protein expression in ocular tissue.

The task underlying the present invention is to make available an improved procedure along with components for treatment of eye diseases.

This problem is solved with the appropriate subject of the patent claims.

The basic idea of the present invention relates to a procedure for specific inhibition of the expression of target genes in cells and tissues of the eye with the following procedural steps:

- introduction of one or more double-strand oligoribonucleotides (dsRNA) outside of the retina, especially of the eye,
- inhibition of the expression of the corresponding mRNA of one or target genes through RNA interference,
- degradation of the corresponding mRNA of one or more target gene
- preparation and maintenance of a test cell or a test tissue, in which the corresponding mRNA of one or more target gene is or are degraded, and
- observation and comparison of the resulting phenotype of the produced test cell or of the test tissue with that of a suitable control cell or of a control tissue, to obtain information about the functions of the gene.

Additionally the present invention relates to a medication or a pharmaceutical compound which contains one or more double-strand oligoribonucleotide (dsRNA), which through RNA interference inhibits the corresponding mRNA of one or more target genes in the expression, whose limited functions are causal for an eye ailment, and which is applied outside the blood-retina barrier, especially outside the eye.

The surprising advantages of the invention are that it could be shown, counter to the prevalent specialists' opinion, that invention-specific agents can overcome the blood-retina barrier as a physiological barrier, so that a systemic or local application outside the eye interior for a targeted treatment of a disease of the ocular fundus, and thus a better therapy, is possible.

Simultaneously the side effects that are connected with a direct application such as through injection, owing to the structure of the human eye, and which, for the affected person, especially with multiple or chronic treatments, are very unpleasant and linked with long-term consequences such as cataracts and glaucoma, are reduced according to the invention.

The solution of the technical problem consists in making available a procedure for specific intervention in diseases of the ocular fundus on the molecular level, without a direct application into the ocular fundus being necessary. With the present invention, the wide therapy field, until now not addressable, or only inadequately, of diseases of the eye's interior and of the ocular fundus is comprehensively developed. The intervention is based on an inhibition of a gene expressed specifically or predominantly in the tissues of the eye or the ocular fundus, characterized in that the agents needed for this can overcome the blood-retina barrier, so that a systemic or local application is possible outside the eye interior for a targeted treatment of a disease of the ocular fundus.

With the same method, simply and rapidly, testimony-credible animal models can be generated, which reproduce the symptoms of the predominantly genetically conditioned diseases of the eye interior. These animal models are suited to initiate the development process of appropriate specific pharmaceuticals for ophthalmology and to validate products.

Further advantages and preferred embodiment forms of the invention are obtainable from the specification.

The solution to the technical problem underlying this invention consists in providing a procedure for specific inhibition of genes whose aberrant functions stand in causal connection with monogene or multifactorally conditioned eye diseases. For this, for example AMD can be counted as a form of a degenerative retinal ailment.

A procedure for specific inhibition of genes through double-stranded oligoribonucleotides (dsRNA) is known from WO 01/75164. The publication of this application is hereby undertaken in the present specification.

In this application will be described that double-stranded oligoribonucleotides (dsRNA) after introduction into the target cells induce the specific degradation of mRNA. The specificity of this

procedure is arranged through the complementarity of one of the two dsRNA strands to mRNA of the target gene.

The process of gene-specific, post-transcriptional switchoff of genes via dsRNA molecules is designated as RNA interference (RNAi). This designation was originally coined by Fire and associates to describe the observation that introduction of dsRNA molecules into the threadworm Caenorhabditis *elegans* blocks the gene expression (Fire et al., 1999). Subsequently RNAi was also shown in plants, protozoa, insects (Kasschau and Carrington 1998) and also recently on mammalian cells (Caplen et al., 2001; Elbashir et al., 2001). The mechanism through which RNAi suppresses gene expression, is not yet fully understood. From investigations on non-mammalian cells it is known that dsRNA molecules are converted through endogenous ribonucleases into small interfering RNA molecules (siRNA molecules) (Bernstein et al., 2001; Grishok et al., 2001; Hamilton and Baulcombe, 1999; Knight and Bass, 2001; Zamore et al., 2000). The 21 to 23-bp-long siRNA molecules are thus the actual mediators for the decomposition of the mRNA of the target gene.

For specific inhibition of a target gene it suffices that the double-stranded oligoribonucleotide has a sequence identical to the target gene with a length from 21 to 23 nucleotides (base pairs).

In the present invention, double-stranded oligoribonucleotides (dsRNA) are used, which after the application overcome the blood-retina barrier, to evoke inhibition of the target genes in the target cells through RNA interference of the corresponding mRNA molecules. The present invention further includes a medication of dsRNA molecules for specific treatment of genetically conditioned eye diseases. With the present invention, the wide therapy field, until now not addressable, or only inadequately, of diseases of the eye's interior and of the ocular fundus is comprehensively developed.

The procedure described in this invention is now distinguished in that for the first time it was shown that dsRNA molecules, preferably of the above-mentioned length, after systemic or local application are detectable outside the eyeball in the interior of the eye. Documentability is based on the specific inhibition of preset target genes in cells or tissues of the eye's interior through RNA interference.

Based on the specific functions of the cells of the retinal tissue and of the RPE it is assumed that genes whose aberrant functions are cause for illnesses of the ocular fundus, are specifically expressed in the tissues and cells of the ocular fundus and thus represented preferred targets for medicinal interventions.

The core of the present invention is surprising in that dsRNA molecules with a length of 21 to 23 nucleotides are in a position, after a systematic application, for example through intravenous injection, to overcome the blood-retina barrier and specifically to inactivate target genes in the tissues of the ocular fundus. This overcoming of the blood-retina barrier is all the more remarkable, since overcoming the blood-brain barrier by dsRNA could not be shown to date in any experiment.

The procedure, which is explained below by embodiment examples, thus is suited for preparation of an animal model with which targets whose limited function is causal for diseases in the eye, can be identified and validated. The procedure in addition is suited for specific intervention in eye diseases at the molecular level, without a direct application being necessary into the ocular fundus. Owing to the specificity of the RNAi for inhibition of genes specifically in the target cells, risk of undesired side effects is small.

This and other embodiment forms are revealed and covered in the present description and in the examples. Literature regarding the materials, procedures, applications and compounds to be used according to the invention can be ordered using public libraries and data bases, for example by means of use of electronic aids. For example, the Medline public data base can be used, which is maintained by the National Center for Biotechnology Information and/or the National Library of Medicine at the National Institutes of Health. Further data bases and internet addresses, like that of the European Bioinformatics Institute (EBI), which is part of the European Molecular Biology Laboratory (EMBL), are known to the specialist and also can be found by use of internet search engines. An overview of patent information in biotechnology and an overview of relevant sources for patent information which is useful for retrospective search and visualization, are described in Berks, TIBTECH 12 (1994), 352-364.

The present publication generally describes the present invention. A comprehensive understanding of the invention can be obtained by reference to the following specific examples, which are made available merely for the purpose of illustration and are not conceived to limit the extent of the invention. The content of all cited references (including literature references, granted patents, published patent applications as they are cited in the text and manufacturers' descriptions and data, etc.) is contained here explicitly through reference; however, this is no concession that any one of these documents is the state of the art for the present invention.

For implementation of the present invention, if not presented otherwise, conventional techniques of cell biology, cell culture, molecular biology, transgene biology, microbiology, recombinant DNA and RNA technology can be used, which belong to the tools of the specialist. Such techniques are explained comprehensively in the literature, see for example Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover, ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U. S. Pat. No. 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription and Translation (B. D. Hames & S. J. Higgins eds., 1984P; Culture of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Ezymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods in Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C.C. Blackwell, eds., 1986).

Embodiment examples

As a model, the inhibition of expression of green-fluorescing protein (eGFP) in the retinal pigment epithelim (RPE) and in the retina of transgene mice (FVB.Cg-Tg(GFPU)5Nagy, The Jackson Laboratory) through dsRNA-molecules is analyzed.

Example 1 describes the specific *post transcriptional gene silencing* through dsRNA for the target gene eGFP in a mouse animal model, whereby the optimal dsRNA concentration for *post transcriptional gene silencing* is to be determined for the systemic application (trial scheme 1). For the implementation, transgene mice, which express the *enhanced* form of the green-fluorescing protein (eGFP) in their body cells, are treated *in vivo* through systemic application with dsRNA oligoribonucleotide molecules for the target gene eGFP. In control animals likewise a systemic treatment takes place with nonspecific dsRNA molecules for the luciferase GL2 gene. For the purpose of *post transcription gene silencing* the non-analgetisized and non-narcotized animals are injected daily (1st treatment day: Day 0, last treatment day: Day 20) with 20, 100 or 200 µg of eGFP-specific dsRNA/kg of body weight (BW) and the control group 200 µg of luciferase-specific dsRNA/kg BW in the tail vein. A control trial animal group treated with ddH2O (daily i.v. injection of 0.5 ml ddH2) into the tail vein) is maintained as well. Each trial animal group consists of 8 animals, the injection volume/injection amounts at maximum to 0.5 ml. On Day 21 the animals are sacrificed via CO2 inhalation.

Expression of the green-fluorescing protein in the eye of untreated and treated mice is investigated immunohistologically (spontaneous eGFP fluorescence: fluorescence microscopic evaluation; eGFP-specific immunofluorescent dyeing: fluorescence microscopic evaluation; eGFP-specific DAB dyeing: optical microscope assessment).

Example 2 describes the specific *post transcriptional gene silencing* through dsRNA for the target gene eGFP in the mouse animal model, whereby the optimal effect time (=time of the maximum *gene silencing* effect) is to be determined after one systemic dsRNA injection i.v. in the tail vein of the *post transcriptional gene silencing* for systemic application (trial scheme 2).

For implementation, transgene mice, who express the *enhanced* form of the green-fluorescing protein (eGFP) in their

body cells, are treated *in vivo* through systemic application with dsRNA oligoribonucleotide molecules for the target gene eGFP. In control animals likewise a systemic treatment takes place with nonspecific dsRNA molecules for the luciferase GL2 gene. For the purpose of *post transcription gene silencing* the non-analgetisized and non-narcotized animals are injected once on Day 0 with 200 µg of eGFP-specific dsRNA/kg of body weight (BW) and the control group 200 µg of luciferase-specific dsRNA/kg BW i.v. in the tail vein. Each trial animal group consists of 8 animals, the injection volume/injection amounts at maximum to 0.5 ml. On Days 2, 3, 5 and 10 after the i.v. injection, the animals are sacrificed via CO2 inhalation.

Expression of the green-fluorescing protein in the eye of untreated and treated mice is investigated immunohistologically (spontaneous eGFP fluorescence: fluorescence microscopic evaluation; eGFP-specific immunofluorescent dyeing: fluorescence microscopic evaluation; eGFP-specific DAB dyeing: optical microscope assessment).

Example 3 describes the specific *post transcriptional gene silencing* through dsRNA for the eGFP target gene in the mouse animal model, whereby the optimal deRNA concentration is to be determined for the local (retrobulbular) application (trial scheme 3). For implementation, transgene mice, who express the *enhanced* form of the green-fluorescing protein (eGFP) in their body cells, are treated *in vivo* through retrobulbular application with dsRNA oligoribonucleotide molecules for the target gene eGFP. In control animals likewise a retrobulbular treatment takes place with nonspecific dsRNA molecules for the luciferase GL2 gene. For the purpose of *post transcription gene silencing* the analgetisized and narcotized animals are injected once)1st treatment day: Day 0) with 20, 100, 200 µg of eGFP-specific dsRNA/kg of body weight (BW) and the control group 200 µg of luciferase-specific dsRNA/kg BW retrobulbularly. Each trial animal group consists of 8 animals, the injection volume/injection amounts at maximum to 0.1 ml. The retrobulbular dsRNA injection takes place both on the left and also on the right eye. On Day 3 the animals are sacrificed via CO2 inhalation.

Expression of the green-fluorescing protein in the eye of untreated and treated mice is investigated immunohistologically (spontaneous eGFP fluorescence: fluorescence microscopic

evaluation; eGFP-specific immunofluorescent dyeing: fluorescence microscopic evaluation; eGFP-specific DAB dyeing: optical microscope assessment).

Example 4 describes the specific *post transcriptional gene silencing* through dsRNA

for the target gene eGFP in the mouse animal model, whereby the optimal effect time (=time of the maximum *gene silencing* effect) is to be determined after one retrobulbular dsRNA injection of the *post transcriptional gene silencing* for the local (retrobulbular) application (trial scheme 4). For implementation, transgene mice, who express the *enhanced* form of the green-fluorescing protein (eGFP) in their body cells, are treated *in vivo* through retrobulbular application with dsRNA oligoribonucleotide molecules for the target gene eGFP. In control animals likewise a retrobulbular application of nonspecific dsRNA molecules for the luciferase GL2 gene. For the purpose of *post transcription gene silencing* the analgetisized and narcotized animals are injected once on Day 0 with 200 µg of eGFP-specific dsRNA/kg of body weight (BW) and the control group 200 µg of luciferase-specific dsRNA/kg BW retrobulbularly. Each trial animal group consists of 8 animals, the injection volume/injection amounts at maximum to 0.1 ml. The retrobulbular dsRNA injection takes place both in the left and also in the right eye. On Days 2, 3, 5 and 10 after the retrobulbular injection, the animals are sacrificed via CO2 inhalation.

Expression of the green-fluorescing protein in the eye of untreated and treated mice is investigated immunohistologically (spontaneous eGFP fluorescence: fluorescence microscopic evaluation; eGFP-specific immunofluorescent dyeing: fluorescence microscopic evaluation; eGFP-specific DAB dyeing: optical microscope assessment).

Example 5 describes the specific *post transcriptional gene silencing* through dsRNA for the eGFP target gene in the mouse animal model, whereby the optimal deRNA effect for *post transcriptional gene silencing* is to be determined for the repeated, local (retrobulbular) application (trial scheme 5). For implementation, transgene mice, who express the *enhanced* form of the green-fluorescing protein (eGFP) in their body cells, are treated *in vivo* through retrobulbular application with dsRNA oligoribonucleotide molecules for the target gene eGFP. In control animals likewise a retrobulbular treatment takes place with nonspecific dsRNA molecules for the luciferase GL2 gene. For the purpose of *post transcription gene silencing* the analgetisized and narcotized animals are injected on Days 0, 7 and 15 (1st treatment day: Day 0, last treatment day: Day 14) with 200 µg of eGFP-specific dsRNA/kg of body weight (BW) and the control group 200 µg of luciferase-specific dsRNA/kg BW retrobulbularly. Each trial animal

group consists of 8 animals, the injection volume/injection amounts at maximum to 0.1 ml. The retrobulbular dsRNA injection takes place both in the left and also in the right eye. On Day 15 the animals are sacrificed

via CO2 inhalation.

Expression of the green-fluorescing protein in the eye of untreated and treated mice is investigated immunohistologically (spontaneous eGFP fluorescence: fluorescence microscopic evaluation; eGFP-specific immunofluorescent dyeing: fluorescence microscopic evaluation; eGFP-specific DAB dyeing: optical microscope assessment).

dsRNA constructs and plasmides:

For the design of the dsRNA molecule, sequences of type AA(N19)TT (where N represents any nucleotide) are selected from the sequence of the target mRNA, to obtain 21 nucleotide (nt) long sense and anti-sense strands with symmetrical two nucleotide long 3' overhangs. In the 3' overhangs, 2'-deoxy-thymindine was used in place of uridine. To guarantee that the dsRNA molecules are directed exclusively against the target gene, the selected dsRNA sequences are tested in a BLAST analysis against the mouse genome. The 21-nt RNA molecules are chemically synthesized and cleaned. For the duplex formation, 100 µg each of the sense and anti-sense oligoribonucleotides are mixed in 10 mM Tris/Hcl, 20 mM NaCl (pH 7.0), heated to 95°C and cooled down over a period of 18 hours to room temperature. The dsRNA molecules are precipitated with ethanol and resuspended in sterile dd H20. the final concentration is 0.5 mg/mL. The integrity and the double-strand character of the dsRNA is verified through gel electrophoresis. Alternatively, dsRNA molecules are ordered from commercial suppliers. The sequences of the target genes and of the corresponding dsRNA molecules are as follows:

GFP dsRNA

DNA target sequence:	5' CGG CAA GCT GAC CCT GAA GTT CAT
	Coding region, 122-142 relative to the first nucleotide of the
	Start codon (Acc. No. X65324)
dsRNA (sense)	5' GCA AGC UGA CCC UGA AGU UCA U
dsRNA (anti-sense)	5' GAA CUU CAG GGU CAG CUU GCC G

Luciferase GL2 dsRNA (non silencing dsRNA, control)

DNA target sequence:	5' AAC GTA CGC GGA ATA CTT CGA

	Coding region, 153-173 relative to the first nucleotide of the Start codon (Acc. No. X65324)
dsRNA (sense)	5' CGU ACG CGG AAU ACU UCG Ad(TT)

dsRNA (anti-sense) 5'UCG AAG UAU UCC GCG UAC Gd(TT)

Analgesia and narcosis of mice:

For systemic application, the animals are secured and the dsRNAs are injected i.v. into the tail vein (maximum injection volume: 0.5 ml), whereby an analgesic or narcosis is avoided, since this would burden the animals more strongly than the i.v. injection itself. For retrobulbular injection (maximum injection volume: 0.1 ml), the animals in fact were subjected to a short-term isofluorane inhalation narcosis and treated analgetically with metamizol sodium. Then the animals are kept in their customary environment in the animal stall. After the completion of the *in vivo* diagnostics (the animal trial completion is to be gleaned from each example 1 – 5) the animals are killed by CO2 inhalation, enucleated, and the eyes are examined histologically (immunohistology).

Investigation of eGFP expression in the retinal pigment epithelium and the retina:

After removal, the eyes are fixed 24 hours long in methyl Carnoy solution. The fixed preparations are then desiccated according to standard methods in a rising isopropanol series and embedded via the intermedium methyl benzoate in paraffin. With the aid of a rotational microtome in standard fashion 5 to 7 µm thick series sections are produced, stretched in a heated water bath and placed on polylysine-coated object carriers. Thereafter the sections are dried for 2 hours in a breeding cabinet at a temperature of 52°C. The dried sections are dewaxed in xylol, transferred into a rising alcohol series and then into Tris/HCl pH 7.4. After the blocking the sections are incubated for 2 hours with the primary anti-eGFP antiserum (polyclonal goat anti-eGFP antiserum, Santa Cruz no. sc-5384). Detection takes place using immunofluorescence dyeing using a Cy2-coupled rabbit anti-goat IgG (Dianova, no. 305-335-045) or optical microscopy through the DAB dyeing method (use of biotinilated anti-goat IgC (Santa Cruz, No. sc-2042), Avidin-D-peroxidase (linaris), DAB reagent kid (Vector Systems)). After counter-dyeing with Haemalaun the samples are embedded and then, using an Eclipse TE-2000-S microscope (Nikon), equipped with a 20x and 40x/1.3 lens, microscope-investigated. The

spontaneous, eGFP-specific fluorescence is analyzed in de-waxed, untreated sections using fluorescence microscopy.

Trial Schemes

Trial scheme 1: systemic siRNA application. Determination of the optimal dsRNA concentration for *post transcriptional gene silencing.*

Group	substance	Number of animals
Untreated animals	ddH2O	8
Negative check 200 µg dsRNA/kg BW	*non silencing* dsRNA (luciferase-specific dsRNA)	8
20 µg dsRNA/kg BW	eGFP-specific dsRNA	8
100 µg dsRNA/kg BW	eGFP-specific dsRNA	8
200 µg dsRNA/kg BW	EGFP-specific dsRNA	8
Animals per experiment		40

Trial scheme 2: systemic siRNA application for determining the optimal effect time of the *post transcriptional gene silencing* (=time of the maximum *gene silencing* effect after one systemic dsRNA injection i.v. in the tail vein) on Day 0.

Group	Substance	Trial ends after	number of animals
Negative check (8 animals per time point)	*non silencing* dsRNA (luciferase-specific dsRNA)	2, 3, 5, 10 days	32
200 µg ds RNA/kg BW	eGFP-specific dsRNA	2 days	8
200 µg dsRNA/kg BW	eGFP-specific dsRNA	3 days	8

200 µg dsRNA/kg BW	eGFP-specific dsRNA	5 days	8
200 µg dsRNA/kg BW	eGFP-specific dsRNA	10 days	8
Animals per experiment			64

Trial scheme 3: retrobulbular siRNA application. Determination of the optimum dsRNA concentration for *post transcriptional gene silencing*.

One-time retrobulbular siRNA injection on day 0, trial ends on Day 3 (BW = body weight)

Group	Left eye	Right eye	Number of animals
Negative check 200 µg dsRNA/kg BW	*non silencing* ds RNA (luciferase-specific dsRNA)	*non silencing* dsRNA (luciferase-specific dsRNA)	8
20 µg dsRNA/kg BW	eGFP-specific dsRNA	eGFP-specific dsRNA	8
100 µg dsRNA/kg BW	eGFP-specific dsRNA	eGFP-specific dsRNA	8
200 µg dsRNA/kg BW	eGFP-specific dsRNA	eGFP-specific dsRNA	8
Animals per experiment			40

Trial scheme 4: retrobulbular dsRNA injection for determining the optimal effect time of the *post transcriptional gene silencing* (=time of the maximum *gene silencing* effect after one retrobulbular dsRNA application on Day 0).

Group	Left eye	Right eye	Trial ends after	Number of animals
Negative check (8 animals per time point)	*non silencing* dsRNA (luciferase-specific dsRNA)	*non silencing* dsRNA (luciferase-specific dsRNA)	2, 3, 5, 10 days	32
200 µg dsRNA/ kg BW	eGFP-specific dsRNA	eGFP-specific dsRNA	2 days	8
200 µg dsRNA/ kg BW	eGFP-specific dsRNA	eGFP-specific dsRNA	3 days	8
200 µg dsRNA/ kg BW	eGFP-specific dsRNA	eGFP-specific dsRNA	5 days	8
200 µg dsRNA/ kg BW	eGFP-specific dsRNA	eGFP-specific dsRNA	10 days	8
Animals per experiment				64

Trial scheme 5: repeated retrobulbular dsRNA injection for determining *post transcriptional gene silencing.*

Retrobulbular injection of 200 µg dsRNA/kg BW on days 0, 7, 14; on Day 15, histological evaluation.

Group	Left eye	Right eye	Injection on day	Number of animals
Negative check (8 animals per time point)	*non silencing* dsRNA (luciferase-specific dsRNA)	*non silencing* dsRNA (luciferase-specific dsRNA)	0, 7, 14	24

200 μg dsRNA/ kg BW	eGFP-specific dsRNA	eGFP-specific dsRNA	0, 7, 14	24
Animals per experiment				48

Literature

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Patent Claims

1. Procedure for specific inhibition of the expression of pre-set target genes in cells and tissues of the eye, characterized in that one or more double-stranded oligoribonucleotides (dsRNA) are applied outside the retinal area of the blood-retina barrier.

2. Procedure according to claim 1, characterized in that

a) one or more double-stranded oligoribonucleotides (dsRNA) are introduced, which arrange for RNA interference of the corresponding mRNA of one or more of the target genes, so that the degradation of the corresponding mRNA in the cell or the tissue occurs, whereby a cell or a tissue is made available, which contains one or more of the introduced dsRNA molecules;

b) the cell or the tissue from a) comes to be maintained under conditions in which the degradation of the mRNA molecules of one or more of the target genes occurs, whereby RNA interference of the mRNA molecules of the corresponding target genes takes place in the cell or in the tissue.

3. Procedure according to claim 1, characterized in that

a) one or more double-stranded oligoribonucleotides (dsRNA) are introduced, which arrange for RNA interference of the corresponding mRNA of one or more gene, so that degradation of the corresponding mRNA in the cell, the tissue or the organism takes places, whereby a test cell, a test tissue or a test organism is made available;

b) the cell or the tissue from a) comes to be maintained under conditions in which the degradation of the mRNA-molecules of one or more of the genes takes place, whereby a test cell or a test tissue comes to be made available in which the mRNA of one or more of the genes is degraded; and

c) observation of the phenotype of the test cell produced in b) or of the test tissue is possible, and, optionally, comparison of the observed phenotype with that of a

suitable control cell or of a control tissue, through which information about the functions of the genes is possible.

4. Procedure according to claims 2 and 3, characterized in that one or more of the target genes code a cellular mRNA.

5. Procedure according to claims 2 and 3, characterized in that the named cells are cells from tissues of the ocular fundus.

6. Procedure according to claims 2 and 3, characterized in that the named cells are cells of the retina.

7. Procedure according to claims 2 and 3, characterized in that the named cells are cells of the retinal pigment epithelium.

8. Procedure according to claims 2 and 3, characterized in that the cells and tissue named in claims 4 to 9 can be assigned to a vertebrate animal.

9. Procedure according to claims 2 and 3, characterized in that the cells and tissue named in claims 4 to 9 can be assigned to a mammal.

10. Procedure according to claims 2 and 3, characterized in that the cells and tissue named in claims 4 to 9 can be assigned to a human.

11. A knockdown organism that is produced by the procedure described in claim 2.

12. A knockdown organism from claim 11, with the organism reproducing an eye disease.

13. A knockdown organism from claim 11, with the organism reproducing a disease of the ocular fundus.

14. A knockdown organism from claim 11, with the organism reproducing a retinal disease.

15. A knockdown organism from claim 11, with the organism reproducing a degenerative retinal disease.

16. A knockdown organism from claims 11 to 15, characterized in that the organism is a vertebrate animal.

17. A knockdown organism from claims 11 to 15, characterized in that the organism is a mammal.

18. Medication or pharmaceutical composition for treatment of an eye disease, characterized in that the application of the medication occurs outside the retina area of the blood-retina barrier.

19. Medication according to claim 18, characterized in that
a) it contains one or more double-stranded oligoribonucleotides (dsRNA), which arrange for RNA interference of the corresponding mRNA of one or more target genes, whose limited functions are causal for an eye disease;
b) it comes to maintain the RNA interference from a) under conditions in which degradation of the mRNA molecules of one or more of the target genes causal for the eye disease takes place.

20. Medication according to claim 18, characterized in that
a) it contains one or more double-stranded oligoribonucleotides (dsRNA), which arrange for RNA interference of the corresponding mRNA of one or more target genes, by which the negative effect of one or more genes limited in their function, that are causal for an eye disease, is efficiently compensated;

b) it comes to maintain the RNA interference from a) under conditions in which the degradation of the mRNA molecules of one or more target genes occurs, whereby the negative effect of one or more

genes limited in their function, which are causal for an eye disease, is efficiently compensated.

21. Medication according to claims 19 and 20 for treatment of a disease of the ocular fundus.

22. Medication according to claims 19 and 20 for treatment of a retinal disease.

23. Medication according to claims 19 and 20 for treatment of a degenerative retinal disease.

24. Procedure according to claims 2 and 3 and claims 19 to 23, whereby one or more of the target genes are specifically expressed in the eye.

25. Procedure according to claims 2 and 3 and claims 19 to 23, whereby one or more of the target genes are specifically expressed in the tissue of the ocular fundus.

26. Procedure according to claims 2 and 3 and claims 19 to 23, whereby one or more of the target genes are specifically expressed in the retina.

27. Procedure according to claims 2 and 3 and claims 19 to 23, whereby one or more of the target genes are specifically expressed in the retinal pigment epithelium.

28. Procedure according to claims 2 and 3 and claims 19 to 23, whereby one or more of the dsRNA molecules employed are between 21 and 23 nucleotides long.

29. Procedure according to claims 2 and 3 and claims 19 to 23, whereby one or more of the dsRNA molecules employed contains a terminal 3'-hydroxyl group.

30. Procedure according to claims 2 and 3 and claims 19 to 23, whereby one or more of the dsRNA molecules employed was chemically synthesized.

31. Procedure according to claims 2 and 3 and claims 19 to 23, whereby one or more of the dsRNA molecules employed represents an analog to a naturally existing RNA.

32. Procedure according to claims 2 and 3 and claims 19 to 23, whereby one or more of the dsRNA molecules employed differs from the corresponding dsRNA analogon from claims 23 to 26 by the additional, deletion, substitution or modification of one or more nucleotides.

33. Procedure according to claims 2 and 3 and claims 19 to 23, whereby one or more of the dsRNA molecules employed inhibits the corresponding gene through transcriptional silencing.

34. Procedure according to claims 2 and 3 and claims 19 to 23, whereby one or more of the dsRNA molecules employed is coded through a vector.

35. Procedure according to claims 2 and 3 and claims 19 to 23, whereby one or more of the dsRNA molecules is introduced via a suitable carrier into cells or tissues of the eye, characterized in that the application occurs outside the blood-retina barrier.

36. Procedure according to claim 35, with the carrier being an adeno-associated virus (AAV).

37. Procedure according to claim 35, whereby the carrier is characterized in that

a) for example a packing of the dsRNA occurs in micelle structures, preferably liposomes;

b) for example a binding of the dsRNA occurs to cationic porphyrines, cationic polyamines, polymeric DNA-binding cations or fusogene peptides;

c) for example an association or packing of the dsRNA occurs into a sheathing protein produced synthetically or derived from a virus like the cytomegalovirus (CMV).

38. Procedure according to claim 35, whereby the dsRNA is present bound to a combination of the carriers named as examples in claim 37.

39. Procedure according to claim 35, whereby the dsRNA is present bound to a combination of the carriers named as examples in claim 37 and to the adeno-associated virus (AAV).

40. Procedure according to claims 35 to 39, whereby the carrier is selected so that the dsRNA molecules after application over a defined time period, are continuously introduced into the target tissue or the target cells.

41. Procedure according claims 35 to 40, whereby the target cells named are cells of the ocular fundus.

42. Procedure according to claims 35 to 40, whereby the target cells named are cells of the retina.

43. Procedure according to claims 35 to 40, whereby the cells named are cells of the retinal pigment epithelium.

44. Procedure according to claims 35 to 40, whereby the target tissue named is tissue of the ocular fundus.

45. Procedure according to claims 31 to 40, whereby the cells and tissues named are cells and tissues of a vertebrate animal.

46. Procedure according to claims 35 to 45, whereby the cells and tissues named are cells and tissues of a mammal.

47. Procedure according to claims 35 to 45, whereby the cells and tissues named are human cells and tissues.

48. In each case at least one component, selected from the group including: pharmaceutical compounds, nucleic acids, organism, host, cell, cell lines,

tissue, organ, medications, carriers, and/or vectors for specific inhibition of the expression of pre-set target genes in cells and tissues of the eye, characterized in that they have one or more double-strand oligoribonucleotides (dsRNA), which are applicable outside the retinal area of the blood-retina barrier.

49. Application of at least one component, selected from the group including: pharmaceutical compounds, nucleic acids, organism, host, cell, cell lines, tissue, organs, medications, carriers, and/or vectors for specific inhibition of the expression of pre-set target genes in cells and tissues of the eye.

50. Component according to claim 48 or utilization of a component according to claim 49 according to one or more embodiment forms of the description.

51. Procedure for identifying a pharmaceutical composition with the steps: making available at least one component from the group including: nucleic acids, organism, host, cell, cell lines, tissue, organs, carriers, and/or vectors for specific inhibition of the expression of pre-set target genes in cells and tissues of the eye, so that one or more double-stranded oligoribonucleotides (dsRNA) are applicable outside the retina area of the blood-retina barrier.

52. Use of the RNA interference method for therapy of the eye.

53. Use of at least one component of the following group, including: nucleic acids, organism, host, cell, cell lines, tissue, organs, carriers, and/or vectors for therapy of the eye using the RNA interference method.